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Evaluation of glycine as an inactivator of glutaraldehyde

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Glycine was evaluated as an inactivator of the sporicidal activity of glutaraldehyde. Spores from glucose depleted cultures of *Bacillus stearothermophilus* grown in a chemically defined medium were used. When glycine is used as an inactivator of glutaraldehyde, it lowers the pH value of the solution. Glycine 1% failed to inactivate 0·5% or higher concentrations of alkaline glutaraldehyde in sporicidal studies. If viable counts cannot be performed within the first hour after inactivation, the concentration of glycine should be at least 2% to inactivate effectively 2% alkaline glutaraldehyde.

In any investigation of the antibacterial or sporicidal effect of a substance on an organism, it is necessary to ensure that the action of the substance is stopped immediately when a sample is taken (Croshaw 1977). This requires the inclusion of a suitable inactivating agent in the recovery medium or tested sample (Russell et al 1979). The use of inactivators in time-survival studies leads to greater reproducibility of results and produces a more quantitative evaluation of the antimicrobial properties of the substance under test.

No satisfactory inactivator has been found for glutaraldehyde. The early proposal by Rubbo & Gardner (1965) that sodium dithionite can be used as an inactivator of glutaraldehyde was later queried by Bergan & Lystad (1971) because the inactivator had an antibacterial effect of its own. Sierra & Boucher (1971) used sodium bisulphite as an inactivating reagent when counting spores treated with glutaraldehyde. However, Bergan & Lystad (1972) and Munton & Russell (1970) found sodium bisulphite to be unsatisfactory; it is a potent bactericide.

Although there are reports indicating that glutaraldehyde neutralization does not require the addition of chemical inactivators, they are open to doubt. Since the methods of centrifuging the glutaraldehyde/cell mixture, followed by washing (Forsyth 1975; Relyveld 1977) and filtering with washing (Miner et al 1977) take time, the adsorbed glutaraldehyde will continue to exert its antimicrobial activity. The accuracy and reproducibility of the result are consequently variable and depend on the time to complete the washing and the concentration of glutaraldehyde used.

More recently Gorman & Scott (1976) evaluated a wide range of potential inactivators of glutaraldehyde in disinfection studies. Of all potential inactivators studied, they found 1% glycine to be the most promising substance in reviving glutaraldehyde-treated *Escherichia coli*. We have evaluated glycine's effect as an inactivator and assessed the concentration required to inactivate 2% glutaraldehyde which is the concentration used for sporicidal activity. Spores from glucose depleted cultures of *Bacillus stearothermophilus* grown in chemically defined media were used to increase reproducibility (Brown & Hodges 1974; Hodges & Brown 1975; Hodges et al 1980).

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MATERIALS AND METHODS

Organism and preparation of spores: Bacillus stearothermophilus NCTC 10,003 grew in 500 ml chemically defined media (CDM) with glucose limiting their exponential growth and the depletion of glucose led to sporulation. The CDM contained (mM): Na_2HPO_4 17·6, KH_2PO_4 7·30, NH_4Cl 4·00, glucose 1·5, L-glutamic acid 2·40, $MnCl_2$ 0·1, $CaCl_2$ 0·1, $MgSO_4$ 0·5 and $FeCl_2$ 0.01, pH 7·0–7·2. Spores were harvested after 60 h incubation at 60 °C in a fermenter, washed in deionized sterile distilled water, resuspended (10^8 spores ml^{-1}) in phosphate-buffered saline (5 mM, pH 7·2) and stored at 4 °C.

Chemicals. Aqueous solutions of glutaraldehyde 4% (pH 3·5) were prepared from a 25% (w/v) solution (Kodak Ltd., Kirkby, Liverpool, U.K.). Before use the solution was made alkaline by the addition of 0·3% (w/v) sodium bicarbonate (Munton & Russell 1970).

Stock solutions of 3% sodium bicarbonate B.P. (BDH) in double distilled water were prepared and sterilized by membrane filtration immediately before use. L-Glycine hydrochloride was purchased from Hopkins and Williams Co., Chadwell Heath, Essex, U.K.

Sporicidal studies

(i) *Glutaraldehyde inactivation by glycine before addition of spores.* In these studies various concentrations of glycine were used for inactivating a mixture of alkaline glutaraldehyde 2% and $MgCl_2$ 0·2 M solution for 20 min before adding spore samples to the liquid. Viable counts were carried out at 2·5, 5 and 7·5 h after the addition of spores, by spreading known volumes of the diluted spore suspension over the surface of 5 replicate dried glucose tryptone plates. The number of colonies developed was counted after incubating the agar plates at 60 °C for 24 h.

(ii) *Glutaraldehyde inactivation by glycine after the addition of spores.* The procedure was similar to that above except that spores were added to the mixture of 2% alkaline glutaraldehyde and 0·2 M $MgCl_2$ solution 30 min before adding the inactivator. Viable counts were carried out at the predetermined intervals after the glutaraldehyde inactivation.

RESULTS

The pH values of 1–11% (w/v) glycine remained the same (Table 1); the pH was 6 when stock glycine solution was diluted by deionized distilled water and it was 6·8 when it was diluted by 5 mM phosphate buffer. When glycine was used at different concentrations to inactivate alkaline glutaraldehyde, different pH values were obtained after inactivation. There was an inverse relationship between the concentration of glycine used and the pH value after inactivation, the pH values vary from 3·8 to 6·0 depending on the final concentration of glycine.

Table 1. pH values of glycine/glutaraldehyde mixtures.

Components in solution	% concn of glycine	pH values (at 20 °C) in Deionized dist. H_2O	pH values (at 20 °C) in 5 mM phosphate buffer
	1·0	6·0	6·8
	2·0	6·0	6·8
	4·0	6·0	6·8
	5·5	6·0	6·8
	11·0	6·0	6·8
2% Glutaraldehyde	0·0	3·5	
2% Glutaraldehyde + 0·3% $NaHCO_3$ + glycine	0·0	7·5	
	0·5	6·0	
	1·0	5·5	
	2·0	4·2	
	4·0	3·8	
2% Glutaraldehyde + 0·6% $NaHCO_3$ + glycine	0·0	7·5	
	0·5	6·8	
	1·0	6·2	
	2·0	5·5	
	4·0	4·5	

When the concentration of sodium bicarbonate was doubled (0·6%) higher pH values were obtained with glycine. From the results glycine can be seen to be both an inactivator of glutaraldehyde and to effect a fall in the pH. These two effects limit the range of glycine concentrations that can be used to inactivate glutaraldehyde, because insufficient glycine would not effectively inactivate glutaraldehyde while excess would lower the pH to a point that could result in a low recovery of glutaraldehyde-treated spores.

In another experiment glutaraldehyde was first inactivated with glycine and spores then added. The results (Fig. 1) suggested that glycine, at concentrations <2%, was not effective for inactivation when counts were carried out within 1 h. A 4% concentration was the optimum for inactivating 2% glutaraldehyde but 2% also gave a good recovery. The slight curve of the revival slope for spores in 2% glutaraldehyde pre-inactivated with glycine could be the result of the time needed for complete inactivation of the glutaraldehyde by glycine. To test this, spores were treated with the 2% alkaline glutaraldehyde for 30 min before glycine at various concentrations was

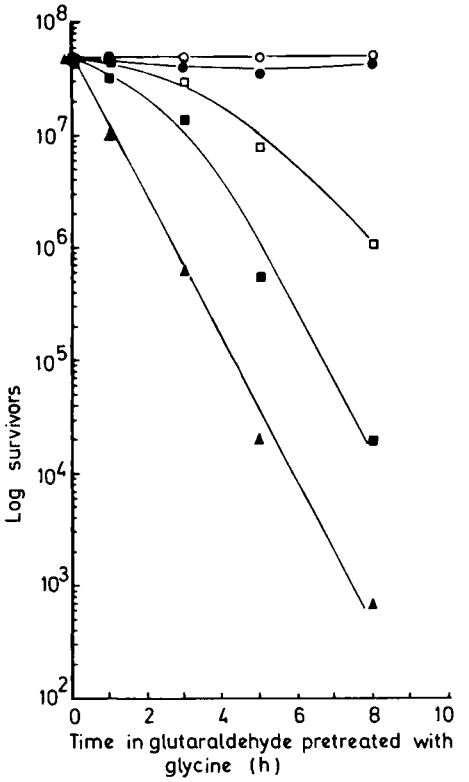

FIG. 1. Inactivation of 2% alkaline glutaraldehyde by glycine. Concentrations (w/v) of glycine used: ▲ 0%, ■ 0·5%, □ 1·0%, ● 2·0%, ○ 4·0%.

added. The results showed that a concentration of glycine above 1% was effective for inactivating 2% alkaline glutaraldehyde. Fig. 2 illustrates the number of spores surviving after 20 min and the inactivation of the glutaraldehyde. Only concentrations of glycine above 1% showed no difference between survivors after 20 min and 8 h of glutaraldehyde inactivation. Presumably, at lower concentrations the residual glutaraldehyde continued to exert its sporicidal effect, thus the longer the time before plating out, the fewer the spores recovered.

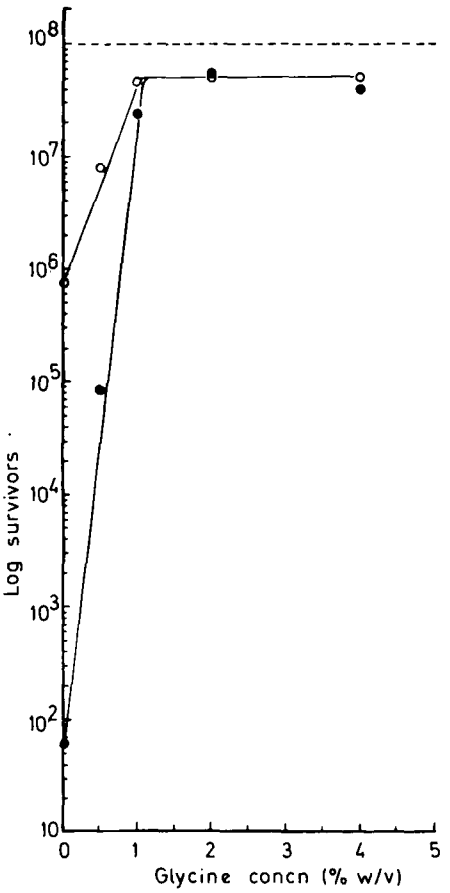

FIG. 2. Recovery of 2% alkaline glutaraldehyde-treated spores with glycine. Symbols - - - Initial viable count of control spores. O——O, Viable count 20 min after and ●——●, 8 h after the inactivation of glutaraldehyde by glycine.

As a concentration of glycine above 1% is necessary to inactivate 2% glutaraldehyde in this system, it remained to compare the recovery of spores following the time course of glutaraldehyde treatment. Survivor counts 3 h after the addition of inactivator to the glutaraldehyde-treated spores (Fig. 3) again showed glycine below 1% to be insufficient to inactivate 2% glutaraldehyde. Although 1% glycine was relatively effective in recovering spores, it was only half as effective as 2% or 4% glycine.

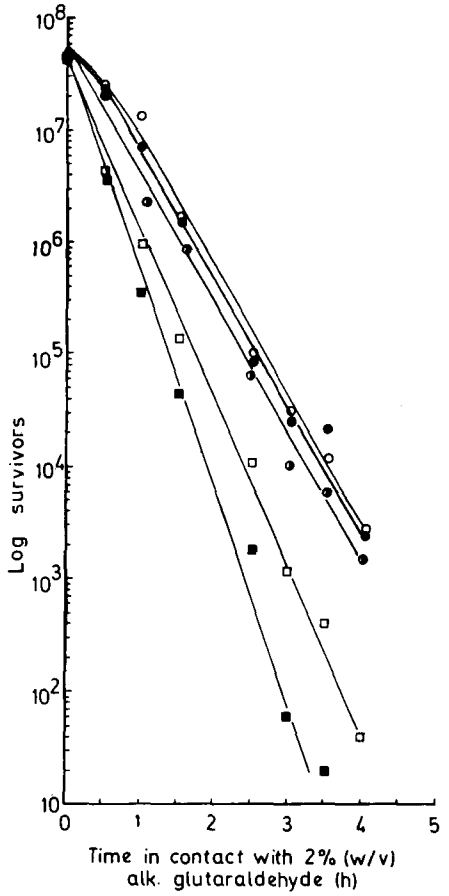

FIG. 3. The effect of different concentrations of glycine on the recovery of 2% alkaline glutaraldehyde treated spores. Concentrations (w/v) of glycine used: ■ 0%, □ 0·5%, ◑ 1·0%, ● 2·0%, ○ 4·0%.

DISCUSSION

Gorman & Scott (1976) suggested glycine to be the most promising inactivator of glutaraldehyde, but gave no quantitative data to substantiate its effectiveness. The use of 1% glycine for inactivating 0·02% alkaline glutaraldehyde may be effective, but Fig. 1 shows that the same concentration does not inactivate the 0·5% or higher concentrations of alkaline glutaraldehyde used in sporicidal studies (Gorman & Scott 1977, 1979). It would appear that the ideal concentration of glycine for effective inactivation of alkaline glutaraldehyde is 2% or higher.

Since glycine is acidic in aqueous solutions, its addition in higher concentrations to alkaline glutaraldehyde will further lower the pH of the solution in which damaged spores are suspended (Table 1). As a consequence spores may not germinate by direct plating unless a weak buffering solution is used. With a weak buffer, glycine concentrations above 2% are suitable for inactivating alkaline glutaraldehyde (Fig. 3). The effectiveness of 2% or higher glycine concentrations in inactivating 2% alkaline glutaraldehyde is further supported by the results of viable counts of spores after 30 min of contact with glutaraldehyde at room temperature 20 °C (Fig. 2). Consistent numbers of spores recovered by direct plating at 20 min and at 8 h after inactivating the action of glutaraldehyde occurred only when using a concentration of glycine above 2% as inactivator (Fig. 2).

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